Fe–S Centers in Lactyl-CoA Dehydratase†

Robert D. Kuchta,‡ Graeme R. Hanson,§,‖ Barton Holmquist,*,§ and Robert H. Abeles‡

Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254, and Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115

Received May 21, 1986; Revised Manuscript Received July 25, 1986

ABSTRACT: Lactyl-CoA dehydratase consists of two enzymes, E1 and E2, and requires catalytic quantities of ATP for activity [Kuchta, R. D., & Abeles, R. H. (1985) *J. Biol. Chem. 260*, 13181–13189]. In contrast to E1, which contains no Fe, E2 contains 8.20 ± 0.04 mol of Fe/mol of E2, one of which can be removed by 1,10-phenanthroline. E2 also contains 7.33 ± 0.68 mol of inorganic sulfur/mol of E2, indicating that at least seven of the Fe atoms are present as Fe–S clusters. E1 and E2 contain <0.14 mol of Cu, Co, Zn, Mn, and Ni/mol of E1 or E2. Both reduced and oxidized E1 are EPR silent over a 10000-G scan range at 4 K, while two signals in E2 are observable at 4 K. Identical spectra were obtained with E2 containing either seven or eight Fe atoms, and both signals were only observable at $T < 30$ K. Signal 1 has axial symmetry with $g_\perp = 2.0232$ and $g_\parallel = 2.0006$. Signal 2 is orthorhombic with $g_1 = 1.982$, $g_2 = 1.995$, and $g_3 = 2.019$. Computer simulation of these spectra with a $S = {}^1/_2$ spin Hamiltonian was used to extract the g matrices. The intensity of both signals decreases when E2 is reduced with $Na_2S_2O_4$. We propose that signal 1 is due to an unusual [4Fe–4S] cluster and signal 2 to a [3Fe–3/4S] cluster. Addition of either acrylyl-CoA or lactyl-CoA dramatically alters signal 2. Thus, substrates bind to E2 and alter the environment of the [3Fe–3/4S] cluster. Neither ATP nor E1 alters the spectrum of E2. O_2 inactivates E2 and causes loss of flavin and the [3Fe–3/4S] cluster. Inclusion of fast protein liquid chromatography during purification of E2 causes partial loss of flavin and alters E2 such that the Fe–S centers become more difficult to oxidize or reduce, but has much less effect upon catalytic activity. The role of the redox-active centers on E2 during the dehydration of lactyl-CoA is not known.

We recently reported the isolation of lactyl-CoA dehydratase, which catalyzes the dehydration of lactyl-CoA to acrylyl-CoA (Kuchta & Abeles, 1985). This elimination is unusual because the abstracted hydrogen is very nonacidic and the nucleophile to be eliminated is α to a carbonyl group. This is in contrast to most enzymatic eliminations where the nucleophile is β to a carbonyl group or other activating group and the abstracted hydrogen is α to the activating group. Very little is known about the mechanism of lactyl-CoA dehydration. Lactyl CoA dehydratase consists of two proteins, E1 and E2,[1] and requires catalytic quantities of ATP. The enzymes exhibit several unusual properties. E1 is rapidly and irreversibly inactivated by O_2 with $t_{1/2} < 60$ s. E2 contains two flavins, but its UV–visible absorption spectrum is nondescript and $Na_2S_2O_4$ reduces the long wavelength absorbance by only 10–15%. Because of the unusual properties of E1 and E2 and the unusual nature of the reaction, we thought the enzymes might contain radicals or other cofactors. We have examined E1 and E2 for the presence of metals and have examined the EPR properties of both proteins. While E1 is EPR silent and does not appear to contain any metals, E2 contains two EPR-active Fe–S clusters.

Experimental Procedures

Materials. All chemicals were reagent grade or better. ATP (Na salt), CoA, and crotonyl-CoA were from Sigma. Certified atomic absorption metal standards were purchased from Fisher. Metal-free H_2O was prepared with a Millipore Milli-Q system. Anaerobic buffers containing no $Na_2S_2O_4$ were prepared by vigorously stirring them in a Coy anaerobic chamber (10% H_2 in N_2) for at least 24 h.

Enzyme Purification. E1 and E2 were purified as described previously (Kuchta & Abeles, 1985) with the following modifications. Enzymes were purified from 45 g of cells. After hydroxyapatite chromatography, if E1 was not at least 90% pure as determined by SDS–PAGE, it was dialyzed for 12 h against 10 mM potassium phosphate, pH 7.5, 1 mg L^{-1} methylene blue, and 0.06% $Na_2S_2O_4$ and the hydroxyapatite chromatography repeated. Typically, 45 g of cells yielded 2.9 mg of E1 with a specific activity of 360 μmol 90 s^{-1} mg of protein^{-1}. Specific activity was determined as described previously (Kutchta & Abeles, 1985).

When E2 was purified from 45 g of cells, it was usually impure when FPLC was the last step, as judged by SDS–PAGE. Therefore, FPLC was omitted and fractions containing E2 were passed through a 3 cm × 1.5 cm hydroxyapatite column equilibrated with 10 mM potassium phosphate, pH 7.5, 1 mg L^{-1} methylene blue, and 0.06% $Na_2S_2O_4$. If at this stage E2 was not >95% pure, the hydroxyapatite step was repeated. Fractions were tested for purity by SDS–PAGE. Fractions containing E2 were detected by absorbance at 400 nm. Fractions from the DE-32 column between 155 and 210 mM phosphate with the ratio A_{400}:mg of protein $mL^{-1} > 0.075$ were saved and further purified by chromatography on Sepharose 6B. Fractions from the Sepharose 6B column with A_{400}:mg of protein $mL^{-1} > 0.13$ were also saved and further purified by chromatography on hydroxyapatite. Typically, 45

† Publication 1601 from the Graduate Department of Biochemistry, Brandeis University (R.D.K. and R.H.A.), and the Center for Biochemical and Biophysical Sciences and Medicine (B.H. and G.R.H.), Harvard Medical School and Brigham and Women's Hospital. This work was supported in part by the Boston Biophysics Research Foundation (to B.H. and G.R.H.), NIH Grant GM12633 (to R.H.A.), and Biochemistry Training Grant GM07596 (to R.D.K.).

‡ Brandeis University.

§ Harvard Medical School and Brigham and Women's Hospital.

‖ Present address: Department of Physics, Monash University, Clayton, Victoria, Australia.

[1] Abbreviations: FPLC, fast protein liquid chromatography; $E2_{FPLC}$, E2 purified with FPLC as the last step; E2, E2 purified with hydroxyapatite chromatography as the last step and *not* subjected to FPLC; EPR, electron paramagnetic resonance spectroscopy; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/86/0425-7301$01.50/0

g of cells yielded 46.5 mg of E2 with a specific activity of 97.5 μmol 90 s^{-1} mg of $protein^{-1}$. Specific activity was determined as described previously (Kuchta & Abeles, 1985). When E2 was purified from 15 g of cells, it was pure when FPLC was the last step in the purification ($E2_{FPLC}$).

E2 was concentrated by ultrafiltration with an Amicon PM-10 membrane. Exposure to O_2 was minimized by flushing the system thoroughly with N_2. E1 was concentrated in the anaerobic chamber by ultrafiltration with an Amicon PM-10 membrane. To prevent O_2 inactivation, solvent was pulled through the membrane with a peristaltic pump.

Protein. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as a standard. In one case, proteins were assayed by the method of Lowry and similar results were obtained.

Enzyme Assays. Acrylyl-CoA hydration assays were performed in the anaerobic chamber as described previously except the assay was performed at 25 °C for only 90 s before it was terminated with concentrated $HClO_4$ (Kuchta & Abeles, 1985).

SDS–PAGE. Denaturing gel electrophoresis was performed as previously described with a 5% stacking and 10% running gel (Laemlli, 1970).

FPLC. E2 was chromatographed on FPLC under aerobic conditions by using the procedure described previously (Kuchta & Abeles, 1985). Immediately after FPLC, E2 was chilled to 4 °C and $Na_2S_2O_4$ added to give a concentration of 5 mM. E2 was then dialyzed against 20 mM potassium phosphate, pH 7.5, and 1 mM $Na_2S_2O_4$.

Quantitation of Flavins on E2. E2 was incubated with O_2 for 24 h at 4 °C. Three volumes of CH_3OH were then added, and precipitated protein was removed by centrifugation. Exposure to light was minimized. Samples were dried with a stream of N_2, and the light yellow residue was dissolved in 0.5 mL of H_2O. Flavins were analyzed by HPLC on a PRP-1 column (Hamilton) using absorbance at 254 nm for detection. The column was equilibrated with 7.5% CH_3CN in 20 mM ammonium acetate, pH 5.8. Following sample injection, the column was washed with 8 mL of 7.5% CH_3CN in 20 mM ammonium acetate, pH 5.8, and then a linear gradient to 50% CH_3CN over 60 mL was applied.

Synthesis of CoA Thio Esters. Acrylyl-CoA and propionyl-CoA were synthesized as described previously (Kuchta & Abeles, 1985). D-Lactyl-CoA was enzymatically synthesized by using the ammonium sulfate precipitated fraction obtained during the purification of E1 and E2. To 1 mL of this fraction (approximately 3 mg of protein), was added 1 mL of 50 mM potassium phosphate, pH 7.5, containing 1 mg L^{-1} methylene blue. This was shaken with air until the methylene blue became oxidized. After approximately 3 min at 0 °C, 20 mg of D-lactate (Li salt), 20 mg of CoA, 1 mg of $Na_2S_2O_4$ and 4 mg of acetyl phosphate were added. This was incubated for 5 min at 37 °C, at which time 20 μL of concentrated $HClO_4$ were added. Precipitated material was removed by centrifugaton and the sample cooled to 0 °C. Lactyl-CoA in the reaction mixture was purified by HPLC by injection of 0.25 mL portions onto a PRP-1 column (Hamilton) with a mobile phase of 3% CH_3CN in 20 mM ammonium acetate, pH 5.8. Fractions containing lactate were detected colorimetrically (Pryce, 1969; Kuchta & Abeles, 1985). A large peak of excess lactate ($k' = 0$–0.5) was detected, as well as a small lactyl-CoA peak ($k' = 0.9$–1.4). Fractions containing lactyl-CoA were pooled, and the CH_3CN was removed under vacuum. The solution was lyophilized to dryness and the residue resuspended in 2 mL of 1 mM HCl. Lactyl-CoA was rechromatographed in eight portions on the PRP-1 HPLC column. Fractions containing lactyl-CoA were detected colorimetrically and pooled. The CH_3CN was removed, and the lactyl-CoA solution was lyophilized to dryness. The residue was taken up in 1–2 mL of 1 mM HCl and stored at −20 °C. The concentration of thio ester was determined by using Ellmans reagent (Fendrich, 1983), and the lactate concentration was determined colorimetrically. These values agreed to within 5%. The UV absorption spectrum was almost identical with that of propionyl-CoA. The purified lactyl-CoA contained 10–15% CoA as determined by HPLC and Ellmans reagent. Yield = 3–5 μmol (13–21%).

UV–Visible Spectroscopy. All UV–visible spectra were measured on a Perkin-Elmer 551 spectrophotometer at 25 °C.

Metal and Sulfur Analyses. Atomic absorption spectroscopy was carried out on a Perkin-Elmer 5000 graphite furnace spectrophotometer by the method of standard additions. Exogenous metals were removed from both E1 and E2 by chromatography on a Sephadex G-25 column (4 mL) made metal free by washing it with 10 mM 8-hydroxyquinoline-5-sulfonic acid followed by metal-free water. Quantitation of Fe in E2 with 1,10-phenanthroline was performed as described previously (Lovenberg et al., 1963).

Inorganic sulfur in E2 was quantitated as described previously (Chen & Mortenson, 1977). The Na_2S standard was standardized with I_2 (Pierce & Haenisch, 1940). E2 was dialyzed for 12 h against anaerobic 10 mM potassium phosphate, pH 7.5, in the anaerobic chamber prior to inorganic sulfur analysis. Oxidized E2 was prepared by incubating it with O_2 for at least 18 h at 25 °C and then dialyzing it for 12 h against 10 mM potassium phosphate, pH 7.5, at 4 °C.

1,10-Phenanthroline Treatment of E2. E2 (3–6 mg mL^{-1}) was incubated with 1 mM 1,10-phenanthroline for 10 min at 4 °C, then dialyzed for 18 h against anaerobic 20 mM potassium phosphate, pH 7.5. Phosphate buffer was made metal free by extraction with a 0.01% solution of diphenylthiocarbazone in CCl_4 (Thiers, 1957). EPR analysis was performed directly on this material, while activity was determined on samples diluted 1:10 with anaerobic 20 mM potassium phosphate, pH 7.5.

EPR Spectroscopy. X-Band EPR spectra were obtained with a Varian E-9 spectrometer equipped with a dual cavity operating in the TE_{104} mode, allowing concurrent magnetic field calibration with Varian strong pitch ($g = 2.0027$). An Air Products LTD-3-110 Heli-Tran liquid-helium transfer line was employed to obtain sample temperatures of 4 K. Measurements were made with nonsaturating microwave power. The EPR spectra of S1 and S2 were examined for power saturation at 0.2 and 200 mW, and no changes in line shape were observed. A modulation amplitude of 10 G and a modulation frequency of 100 kHz were used unless noted otherwise. The spectrometer was interfaced to an Apple IIe microcomputer, allowing signal averaging and data manipulation procedures to be routinely performed. The computer simulation studies were performed on a Vax 11/780 using second-order perturbation theory for a solution to the $S = {}^1/_2$ spin Hamiltonian: $\mathcal{H} = \beta \mathbf{H} \cdot g - \mathbf{S}$.

Copper(II) ethylenediaminetetraacetic acid was used as a standard for the spin-quantitation measurements. The EPR spectra were doubly integrated (Wyard, 1965) and the electron spin(s) of the unknown species were calculated from

$$N_u = N_k \frac{A_u g_{1k}^2 MA_k RG_k P_k^{1/2} S_k(S_k + 1) SW_k^2}{A_k g_{1u}^2 MA_u RG_k P_u^{1/2} S_u(S_u + 1) SW_u^2} \quad (1)$$

where the subscripts (u, k) refer to the unknown and standard

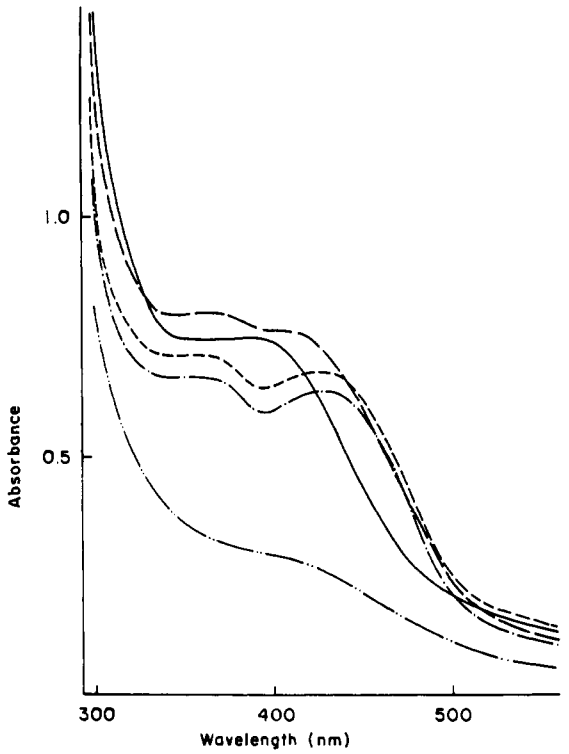

FIGURE 1: Effect of O_2 on the UV–vis spectrum of E2. E2 (3.49 mg mL^{-1}) was dialyzed against anaerobic 20 mM potassium phosphate, pH 7.5. The spectrum was determined (—), and then the sample was made aerobic. Spectra determined immediately after addition of O_2 (– –), 3 h later (- - -) and 6 h later (–·–) are shown. The sample was removed from the cuvette and chromatographed on a 5 mL Sephadex G-25 column in 20 mM potassium phosphate, pH 7.5 and the spectrum determined (–··–).

samples, respectively, N is the number of spins, A is the doubly integrated area, MA is the modulation amplitude. RG is the receiver gain, P is the microwave power, S is the electron spin, and SW is the scan width.

E1 and E2 were concentrated and dialyzed in the appropriate buffer for at least 12 h. All samples were prepared in the anaerobic chamber and sealed with a septum and Parafilm to prevent exposure to O_2. Samples were frozen in liquid N_2 within 2 min of preparation. All additions were made through the septum.

Protein concentrations are as noted in the figure captions. Samples with ATP·Mg contained 0.2–0.4 mM ATP and 15 mM $MgSO_4$. Samples with E1 and E2 contained 6–7 μM E1 and 10–20 μM E2.

Results

Stability of E2. E2 remains active for at least 4 months when stored anaerobically with 4 mM $Na_2S_2O_4$ at 4 °C. No loss of activity was observed when E2 was dialyzed against anaerobic 20 mM potassium phosphate, pH 7.5, and stored at 4 °C for 1 week, indicating that $Na_2S_2O_4$ is not required for short term stability. Incubation of E2 with O_2 for 24 h at 25 °C caused complete inactivation.

Spectral Properties and Metal Content of E2. The UV–visible spectrum of E2 is shown in Figure 1. In anaerobic 20 mM potassium phosphate, pH 7.5, ϵ_{400} = 23 700 M^{-1} cm^{-1}. Reduction with 1 mM $Na_2S_2O_4$ decreased the absorbance at wavelengths >370 nm by approximately 12%, but did not affect the shape of the spectrum. The UV–visible spectrum of E2 was unchanged by the addition of ATP·Mg (0.17 mM ATP, 10 mM $MgSO_4$), lactyl-CoA (78 μM, ±ATP·Mg) and crotonyl CoA (148 μM, ±ATP·Mg), a poor substrate for lactyl CoA dehydratase (R. Kuchta, unpublished results).

Exposure to O_2 causes an immediate increase in the absorbance of E2 between 300 and 450 nm (Figure 1), consistent with the oxidation of a flavin. With longer exposure to O_2 the absorbance decreased and features with λ_{max} at 430 and 360 nm appeared, suggesting that flavin was dissociating from the protein. Sephadex G-25 chromatography of O_2-treated E2 separated the species with λ_{max} at 430 and 360 nm from the protein (Figure 1). The separated chromophore eluted in the low molecular weight fraction of the G-25 column and had absorbance maxima at 445, 375 and 263 nm, consistent with it being a flavin.

E2 was analyzed by atomic absorption spectroscopy and found to contain 8.20 mol of Fe/mol of E2. When the Fe content was determined by using 1,10-phenanthroline, 8.13 mol of Fe/mol of E2 was found. After treatment with 1 mM 1,10-phenanthroline, the protein contained 7.16 mol of Fe/mol of protein, indicating the presence of one labile Fe atom. Fe analysis using 1,10-phenanthroline confirmed the loss of Fe. The loss of Fe did not affect catalytic activity.

The lack of any absorbance maxima between 500 and 650 nm suggested the Fe atoms in E2 are present as non-heme Fe in Fe–S clusters. This was confirmed by inorganic sulfide analysis. Prior to measurement the enzyme was dialyzed against anaerobic 20 mM potassium phosphate, pH 7.5, to remove any $Na_2S_2O_4$ breakdown products that interfere with the assay (Chen & Mortenson, 1977). Analysis showed that 7.38 ± 0.68 mol of S^{2-}/mol of E2 was present, suggesting at least seven Fe are present in Fe–S clusters.

In addition to causing the dissociation of flavin from E2, O_2 also results in the loss of the [3Fe–3/4S] cluster (cf. EPR results). This was shown by incubating E2 with O_2 for 24 h at 25°C followed by chromatography on a metal-free Sephadex G-25 column. The eluted protein contained 4.09 mol of Fe/mol of protein as determined by atomic absorption spectroscopy, 3.76 mol of Fe/mol of protein as determined by the 1,10-phenanthroline method, and 3.7 mol of S^{2-}/mol of protein. Extended exposure to O_2 for 2 weeks at 4 °C caused no further dissociation of Fe.

$E2_{FPLC}$. E2 was also purified with FPLC as the last step ($E2_{FPLC}$). This material differed from E2 in several respects. In anaerobic 20 mM potassium phosphate, pH 7.5, ϵ_{400} = 18 300 M^{-1} cm^{-1} for $E2_{FPLC}$, 22% less than E2, although the spectrum of each had similar line shapes. $Na_2S_2O_4$ (1 mM) affected $E2_{FPLC}$ and E2 similarly. $E2_{FPLC}$ contained 40% less flavin than E2, consistent with the lower absorbance at 400 nm. The relative amounts of FMN and riboflavin were approximately equal, indicating that FPLC does not cause preferential loss of either flavin. Catalytic activity was decreased 27% compared to that of E2 in standard acrylyl-CoA hydration assays. The Fe and S^{2-} content and effect of O_2 on the Fe content of $E2_{FPLC}$ were the same as for E2.

$E2_{FPLC}$ was analyzed for other metals by atomic absorption spectroscopy. There were <0.05 mol of Ni, <0.05 mol of Zn, 0.14 mol of Cu, <0.05 mol of Co, and <0.05 mol of Mo/mol of $E2_{FPLC}$.

EPR of E2. EPR spectroscopy was used to obtain more information on the type and catalytic significance of the Fe–S clusters in E2. Initial experiments were performed on $E2_{FPLC}$. Figure 2a shows the spectrum of $E2_{FPLC}$ dialyzed for at least 2 days against buffer containing 20 mM $Na_2S_2O_4$. The signal intensity was equivalent to 0.26 unpaired electron. The signal (S1) has axial symmetry with $g_\perp$ = 2.0232 and $g_\parallel$ = 2.0006. No other signals were observed when the sample was scanned over 10 000 G. At 30 K, the signal was not observable, in-

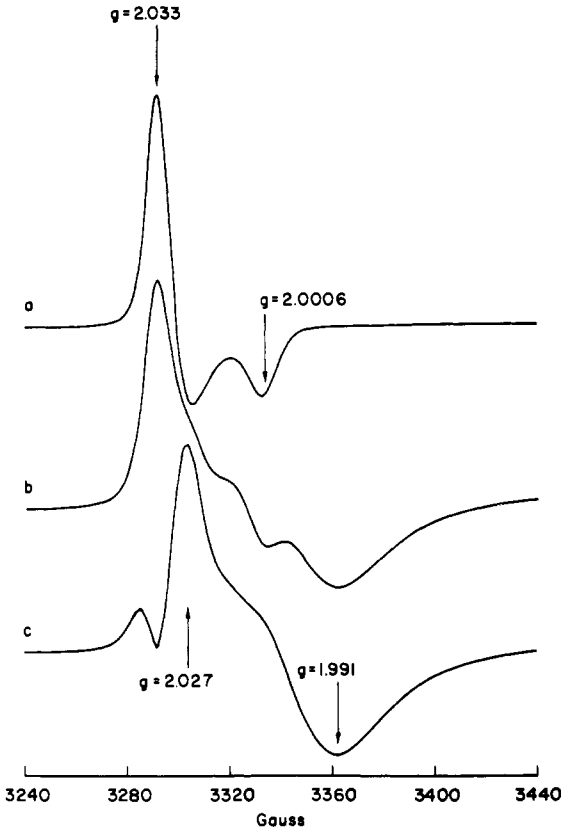

FIGURE 2: EPR spectra of $E2_{FPLC}$. (a) EPR spectrum of $E2_{FPLC}$ (13.0 mg mL^{-1}) dialyzed against 25 mM $Na_2S_2O_4$ and 50 mM potassium phosphate, pH 7.5, for 2 days. The microwave frequency was 9.360 GHz. (b) Spectrum of sample used for spectrum a after incubation with O_2 for 2 h at 20 °C. (c) Computer subtraction of spectrum a from spectrum b to yield the spectrum of S2. The small feature near 3285 G is an artifact of the subtraction process.

dicating a fast relaxation time (T_1). This is typical of Fe–S clusters, particularly those with three Fe and four Fe atoms, as are g values near 2. However, the spectrum is very unusual for an Fe–S cluster in that it has axial symmetry with $g_\perp > g_\parallel$ and that there are no features near $g = 1.94$. It is unlikely the signal is from flavin radicals, since they are generally isotropic and observable at much higher temperatures.

$E2_{FPLC}$ was oxidized by shaking with air until the sample changed from dull yellow, typical of reduced E2, to a brighter yellow, typical of oxidized E2. This caused no change in the EPR spectrum. Upon extended exposure to air (2 h at 20 °C), a second signal, S2, was observed (Figure 2b). The line shape of S2 (Figure 2c) was determined by subtraction of S1. S2 is orthorhombic with $g_{av} = 1.999$. The g matrix and line shape of S2 are very similar to those of other [3Fe–3/4S] clusters (Beinert & Thomson, 1983). The effect of temperature on S2 was identifical with its effect on S1, suggesting S2 is also an Fe–S cluster and not a flavin radical.

The effect of $Na_2S_2O_4$ on S2 was examined by adding 10 mM $Na_2S_2O_4$ to $E2_{FPLC}$ exhibiting S1 and S2 and rapidly freezing the sample. This caused no change to the EPR spectrum.

The EPR spectra of either reduced or oxidized $E2_{FPLC}$ showed no evidence of flavin radicals. Samples of $E2_{FPLC}$ ($\pm O_2$, ±25 mM $Na_2S_2O_4$) were scanned with varying microwave powers, and no other signals were observed.

Subsequently, experiments were performed with E2 and an important difference was noted. Whereas $E2_{FPLC}$ reduced with 25 mM $Na_2S_2O_4$ exhibited a significant amount of S1, E2 reduced with 25 mM $Na_2S_2O_4$ exhibited a very weak signal

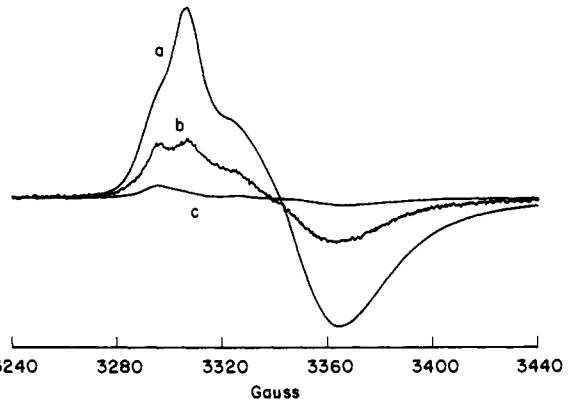

FIGURE 3: EPR spectra of E2. E2 (4.1 mg mL^{-1}) was dialyzed against anaerobic 20 mM potassium phosphate, pH 7.5 containing (a) no $Na_2S_2O_4$, (b) 1 mM $Na_2S_2O_4$, and (c) 25 mM $Na_2S_2O_4$. The microwave frequency was 9.348 GHz.

that consisted of S1 and S2 (Figure 3c). EPR spectra of E2 in buffer containing progressively less $Na_2S_2O_4$ show progressively more S1 and S2 (Figure 3). The signal intensity of E2 in anaerobic buffer (no $Na_2S_2O_4$) was equivalent to 1.1 unpaired electrons. Further oxidation of the $Na_2S_2O_4$-free sample by thawing the sample and shaking with air for approximately 30 s resulted in a 14% increase in the signal intensity. S1 and S2 from E2 showed the same temperature dependence as those from $E2_{FPLC}$, and no other signals were observed when the sample was scanned over 10 000 G.[2]

To determine if the differences between $E2_{FPLC}$ and E2 were due to the FPLC procedure, E2 was subjected to FPLC. This resulted in E2 behaving much like $E2_{FPLC}$. The EPR spectrum of E2 subjected to FPLC and dialyzed for 24 h in buffer containing 25 mM $Na_2S_2O_4$ consisted of S1 and a small amount of S2. Dialysis for 1 week against buffer containing 25 mM $Na_2S_2O_4$ removed the small amount of S2. The signal intensity of E2 in anaerobic buffer (no $Na_2S_2O_4$) was 50% more intense than that of FPLC-treated E2. Incubation with 25 mM $Na_2S_2O_4$ reduced the signal intensity of E2 much more than that of FPLC-treated E2. Additionally, briefly exposing partially reduced E2 to O_2 increased the signal intensity 310%, whereas briefly exposing partially reduced FPLC-treated E2 to O_2 increased the signal intensity only 12%. Thus, FPLC dramatically alters the ability of the Fe–S centers to respond to O_2 and $Na_2S_2O_4$.

Exposure of E2 to O_2 for 18 h at 25 °C results in a form of E2 that contains only four Fe and four S^{2-} and causes >90% of S2 to disappear. This indicates that S2 is O_2 labile and provides further evidence that S1 is due to either a [4Fe–4S] or two identical [2Fe–2S] clusters as this E2 contains only four Fe atoms. Treatment of this material with 10 mM $Na_2S_2O_4$ for 30 min at 20 °C caused no change in the spectrum. Thus, the Fe–S cluster(s) can no longer be easily reduced.

$K_3[Fe(CN)_6]$ is a mild oxidant capable of oxidizing Fe–S clusters. The effect on the EPR spectrum of E2 when $K_3[Fe(CN)_6]$ is added anaerobically is shown in Figure 4. The increased intensity of S1 and the decrease in intensity of S2 indicate that both Fe–S clusters are further oxidized. In addition to this there are new features at $g = 2.100$, 2.078, and 2.009. The isotropic signal at $g = 2.009$ can be attributed to the excess of $K_3[Fe(CN)_6]$ present. The remaining features at $g_1 = 2.100$, $g_2 = 2.078$, and $g_3 =$ (unknown) can be at-

[2] The relative amount of S1 and S2 observed with E2 in anaerobic buffer containing no $Na_2S_2O_4$ varied considerably between enzyme preparations, from approximately 25% to 75% S1. The cause of this variation is unknown.

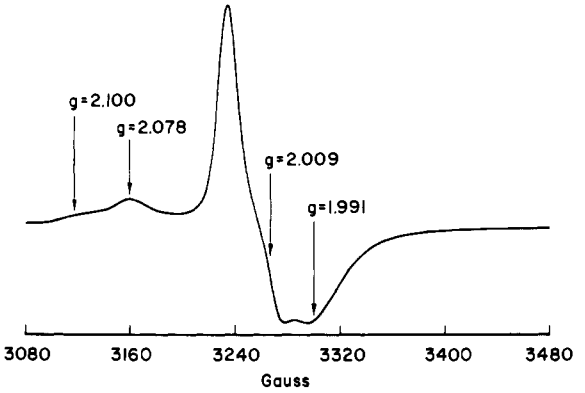

FIGURE 4: Effect of $K_3[Fe(CN)_6]$ on E2: EPR spectrum of E2 (3.51 mg mL^{-1}) in anaerobic 20 mM potassium phosphate, pH 7.5, after addition of 5 equiv of $K_3[Fe(CN)_6]$. The microwave frequency was 9.187 GHz.

Table I: g and Line Width Matrices Determined from Computer Simulation Studies[a]

S1	$g_\perp = 2.0232$		$g_\parallel = 2.0006$
	$W_\perp = 8.5$		$W_\parallel = 7.0$
	$(\Delta g/g)_\perp = 0.001$		$(\Delta g/g)_\parallel = 0.0015$
S2	$g_1 = 1.982$	$g_2 = 1.995$	$g_3 = 2.019$
	$W_1 = 52.7$	$W_2 = 39.1$	$W_3 = 17.0$
	$(\Delta g/g)_1 = 0.0$	$(\Delta g/g)_2 = 0.0$	$(\Delta g/g)_3 = 0.0$

[a] The units for the line widths (W) are MHz.

tributed to a new species whose origin is currently under investigation.

Computer Simulation of the EPR Spectra S1 and S2. Computer simulation of the EPR signals S1 and S2 was performed in order to determine the g matrices and the electron spin quantum number for both of the Fe–S centers. Assuming the Fe–S centers giving rise to S1 and S2 have a single unpaired electron, then S1 and S2 can be simulated by using the following equation (Pilbrow, 1984):

$$S(\nu_c,B) = C \sum_{\theta=0}^{\pi/2} \sum_{\phi=0}^{\pi/2} g_1^2 f(\nu_c - \nu_0[B],\sigma_\nu)\Delta(\cos\theta)\, d\phi \quad (2)$$

In this equation g_1^2 is the powder-averaged expression for the transition probability (Pilbrow, 1969) and f is the line shape function assumed to be Gaussian. In the line shape function f, $h\nu_0[B]$ is the actual energy difference between the energy levels evaluated with second-order perturbation theory (Toy et al., 1971). The calculation of the line shape function in energy units removes the need for the $1/g$ factor. This was introduced by Aasa and Vanngard (1975) to take into account the differences in the transition probability between frequency and field swept experiments. The effect of g strain (Hagen, 1981) on the line widths was calculated from the equation (Pilbrow, 1984)

$$\sigma_\nu = \{\sigma_R^2 + (\Delta g/g\nu_0(B))^2\}^{1/2} \quad (3)$$

where σ_R is the residual line width, assumed to be Gaussian due to dipolar broadening etc., Δg is the half-width of the Gaussian distribution of the g values, and $\Delta g/g$ is the g-strain parameter. Finite difference differentiation of the absorption spectrum was used in the computer stimulation program (EPR50F) since the experimental conditions require the use of a finite field modulation amplitude.

The g and line width matrices together with the values of $\Delta g/g$ are listed in Table I, and the simulated spectra of S1 and S2 are shown in Figure 5.

Clearly, the resonant field positions of S1 and S2 have been accurately simulated by using a $S = {}^1/_2$ spin Hamiltonian,

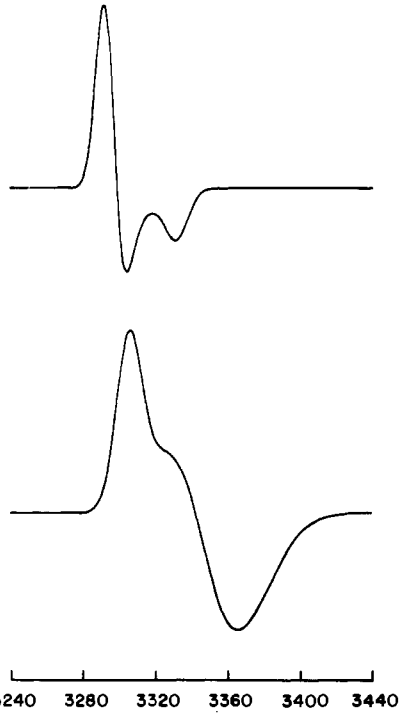

FIGURE 5: Simulated spectra of S1 (top) and S2 (bottom) (see Experimental Procedures).

implying that each chromophore giving rise to S1 and S2 has one unpaired electron. The simulation of the line shapes is not as accurate because of the inadequacies of the g-strain formalism.

Role of Fe–S Clusters in Catalysis. Lactyl-CoA dehydratase requires E1, E2, and ATP·Mg for catalytic activity. Both $E2_{FPLC}$ and E2 were prepared in buffer containing 0 or 1 mM $Na_2S_2O_4$, and either E1, ATP·Mg, or E1 + ATP·Mg was added. None of these additions caused any detectable changes to their respective EPR spectra.[3]

When lactyl-CoA and acrylyl-CoA, the substrates for lactyl-CoA dehydratase, were added anaerobically to E2, the EPR spectra showed marked changes (Figure 6c,d) from those of the native enzyme. Crotonyl-CoA, a very poor substrate, and propionyl-CoA, which is not a substrate, also caused changes in the EPR spectra, which were different from that of either lactyl-CoA or acrylyl-CoA (Figure 6e,f). CoA only changed the spectrum of E2 slightly, indicating the acyl moiety is the primary effector of the observed spectral changes. It is apparent from Figure 6 that S1 remains essentially unaffected while S2 undergoes dramatic changes. Confirmation of this was provided by observing the changes to the EPR spectrum of E2 in 25 mM $Na_2S_2O_4$ when substrates or pseudosubstrates were added. Addition of propionyl-CoA or crotonyl-CoA did not affect S1, indicating that they do not alter the environment of the Fe–S cluster associated with S1.

Addition of $Na_2S_2O_4$ (1 mM) did not alter the interactions of any of the CoA thio esters with the Fe-S clusters. The spectra were less intense, but the line shapes were identical with those obtained in the absence of $Na_2S_2O_4$.

Removal of One Fe with 1,10-Phenanthroline. 1,10-Phenanthroline removes one Fe from E2, but does not affect its catalytic activity. Similarly, the EPR spectra of E2 containing either seven or eight Fe atoms were identical. Further, the spectra obtained after addition of acrylyl-CoA were also

[3] Results of experiments involving E2 + E1 (±ATP·Mg) were inconclusive. The spectra were too weak to be analyzed. This was probably due to the 3–5 mM $Na_2S_2O_4$ in the E1 reducing the E2.

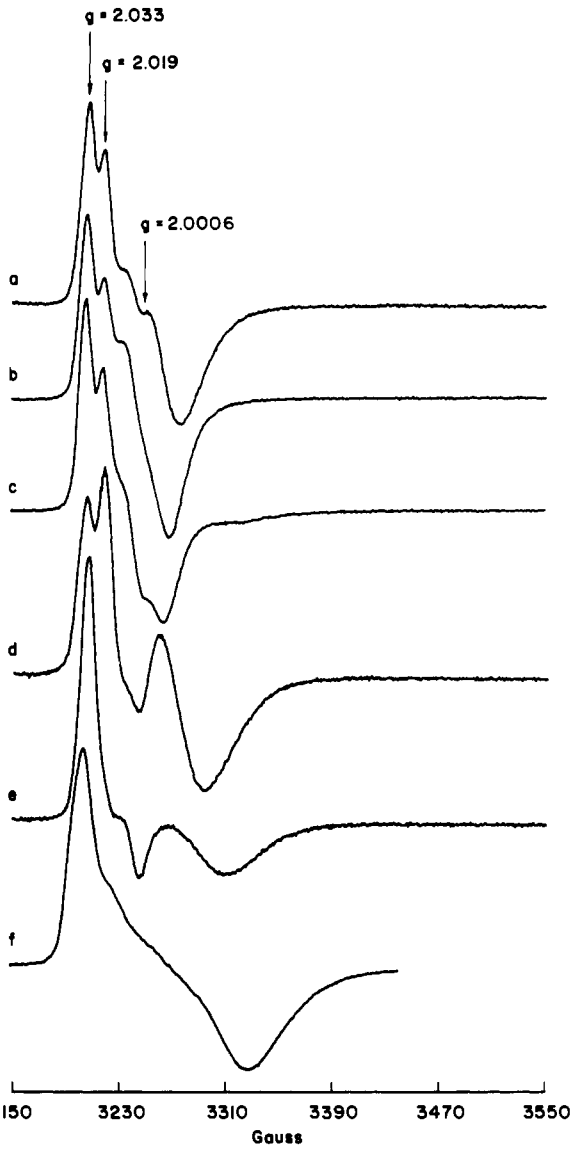

FIGURE 6: Effect of CoA thio esters on E2. E2 (3.12 mg mL^{-1}) was dialyzed against anaerobic 20 mM potassium phosphate, pH 7.5. EPR spectra of E2 were determined with the following additions: (a) none; (b) 0.12 mM CoA; (c) 0.09 mM lactyl-CoA; (d) 0.13 mM acrylyl-CoA; (e) 0.13 mM crotonyl-CoA; (f) 0.05 mM propionyl-CoA. The microwave frequency was 9.187 GHz.

identical, indicating that the Fe removed by 1,10-phenanthroline is not required for activity and is not part of either Fe–S cluster. The absence of any EPR signals at g = 4 or 6 suggests it is present in E2 as a ferrous (Fe^{2+}) ion (S = 2).

Metals Analyses and EPR Spectroscopy of E1. Atomic absorption spectroscopy of E1 indicated the presence of <0.1 mol of Fe, Cu, Ni, Mo, Co, and Zn/mol of E1. Thus, it is likely that E1 does not require any of the above metals for activity.

The absence of any EPR-active species from either oxidized or reduced E1 (40 μM) suggests that its extreme oxygen sensitivity is not due to the presence of a radical.

Discussion

E2 of lactyl-CoA dehydratase contains two Fe–S clusters that give rise to two EPR signals S1 and S2. Several lines of evidence indicate that S1 is due to a [4Fe–4S] cluster, while S2 is due to a [3Fe–3/4S] cluster: A form of E2 can be obtained (O_2 exposure) that contains only 4Fe and 4S and which only retains S1. At $T > 30$ K S1 is no longer observable, implying that the Fe–S cluster has a fast relaxation time T_1. This is typical of 4Fe–4S, [3Fe–3/4S] and Fe–4S clusters and atypical for 2Fe–2S clusters whose EPR spectra can often be observed at 77 K.

Satisfactory computer simulation of S1, assuming a $S = {}^1/_2$ spin Hamiltonian and the absence of signals at $g = 4.3$, rules out the possibility of a rubredoxin type [Fe–4S] ($S = {}^5/_2$) center (Dowing & Gibson, 1969; Orme-Johnson & Sands, 1973).

The EPR-active species giving rise to S1 is paramagnetic when oxidized and diamagnetic when reduced. While both [4Fe–4S] (e.g. high-potential iron protein) and [3Fe–3/4S] clusters are paramagnetic when oxidized ($S = {}^1/_2$) and diamagnetic when reduced (S = 0 and 2 respectively), all known [2Fe–2S] centers are paramagnetic when reduced ($S = {}^1/_2$) and diamagnetic when oxidized (S = 0) (Thomson, 1985). The line shape of S1 is inconsistent with the EPR spectra of known [3Fe–3/4S] clusters (Beinert & Thomson, 1983).

A [4Fe–4S] cluster is most consistent with our data. The spectrum of this center is, however, unusual because it is axial with $g_\perp = 2.0232 > g_\parallel = 2.0006$. We know of no other Fe–S cluster with this type of spectrum.

The EPR-active species giving rise to S2 is most likely an [3Fe–3/4S] cluster. S2 is only observable at temperatures below 30 K, implying that the Fe–S cluster has a fast relaxation time T_1. All known [3Fe–3/4S] clusters exhibit orthorhombic EPR spectra with $g_{av} = 2.0$ and are paramagnetic when oxidized [$3Fe–3/4S^{3+/+}$] and diamagnetic when reduced [$3Fe–3/4S^{2+/0}$]. S2 exhibits all of these properties. Satisfactory computer simulations of S2 using a $S = {}^1/_2$ spin Hamiltonian indicate the EPR-active species has one unpaired electron as expected for an [3Fe–3/4S] cluster in its oxidized state.

E2, containing four Fe atoms as a result of O_2 exposure, has an EPR spectrum consisting of only S1, whereas E2 that contains seven Fe atoms has an EPR spectrum consisting of S1 and S2. Thus, the loss of three Fe atoms results in the loss of S2, consistent with S2 being due to a [3Fe–3/4S] center.

Inorganic sulfide analysis of E2 indicated the presence of 7.38 ± 0.68 mol of S^{-2}/mol of E2. The experimental error associated with the sulfide determination (Wahl & Rajagopalan, 1982; Beinert & Thomson, 1983) makes it impossible to distinguish between 3 and 4 mol of S^{2-} for the 3Fe cluster. Furthermore, $[3Fe–4S]^{3+}$ clusters (e.g. inactive aconitase) and $[3Fe–3S]^{+}$ clusters (e.g. glutamine synthase) exhibit similar EPR properties (Beinert & Thomson, 1983).

$Na_2S_2O_4$ is a powerful reducing agent that rapidly reduces most Fe–S clusters (Latimer, 1964). Surprisingly, the Fe–S clusters in E2 are only partially reduced by 1 mM $Na_2S_2O_4$. Even in the presence of 25 mM $Na_2S_2O_4$, a small amount of the Fe–S clusters remain oxidized. The fact that $Na_2S_2O_4$ only partially reduces the Fe–S clusters suggests that either they have extraordinarily low oxidation potentials or they are only partially accessible to solvent.

FPLC of E2 causes a partial loss of activity and greatly alters the properties of the Fe–S centers. Thus, it appears that this procedure partially traps the [4Fe–4S] cluster in the oxidized state and the [3Fe–3/4S] cluster in the reduced state, and greatly reduces the rate at which $Na_2S_2O_4$ reduces or O_2 oxidizes either cluster. These changes may be due to the loss of flavin caused by FPLC, particularly if the flavins and the Fe–S centers are involved in an electron-transfer chain. Alternatively, FPLC may cause a large conformational change in E2 that results in the centers becoming much less accessible to solvent, or FPLC changes the oxidation potentials such that

$Na_2S_2O_4$ ($E_o' = -1.12$ V) cannot readily reduce and O_2 ($E_0' = +0.81$ V) cannot rapidly oxidize E2 (Latimer, 1964).

If both Fe–S centers are oxidized, the signal intensity should be equivalent to 2 unpaired electrons. Maximally, however, the signal intensity was equivalent to 1.2 unpaired electrons. This discrepancy is probably a result of partially trapping E2 in the reduced state during purification. In addition to this problem, it is quite difficult to obtain reliable numbers from spin quantitation measurements, since it is difficult to maintain exact experimental parameters for both the unknown and standard samples. Also, differences between frequency and field swept experiments (Pilbrow, 1984) can lead to errors in the transition probability g_1^2 (eq 2).

While lactyl-CoA and acrylyl-CoA interact with the [3Fe–3/4S] center when it is oxidized, we do not know if they interact with the center when it is reduced, or if E2 is active when the [3Fe–3/4S] cluster is oxidized. During catalysis the [3Fe–3/4S] cluster is reduced, either by trapping in the case of $E2_{FPLC}$ or by reduction by $Na_2S_2O_4$ in the case of E2 (enzyme assays contain 4 mM $Na_2S_2O_4$).

The EPR spectrum of E2 is perturbed by addition of lactyl-CoA and acrylyl-CoA, the normal substrates for lactyl-CoA dehydratase. CoA thio esters (crotonyl-CoA or propionyl-CoA) bind to E2 and alter the environment of the [3Fe–3/4S] cluster. The spectral perturbations resulting from the addition of substrates or CoA thio esters to E2 my arise from either the binding of the substrate to the [3Fe–3/4S] or a conformational change resulting from the binding of the substrate close to this cluster. The reaction catalyzed by lactyl-CoA dehydratase is not a redox reaction. Thus, E2 may be similar to active aconitase, which contains a [4Fe–4S] center that is the substrate binding site (Emptage, et al., 1983), or the Fe-S centers may be serving a purely structural role, as appears likely in glutamine phosphoribosyl pyrophosphate amido transferase from *Bacillus subtilis* (Vollmer et al., 1983).

Lactyl-CoA dehydratase catalyzes a dehydration reaction for which it is difficult to envisage a mechanism involving typical acid–base catalysis, making it attractive to consider a mechanism that proceeds via radical intermediates and/or redox processes as previously proposed (Kuchta & Abeles, 1985). The flavins and Fe–S centers may mediate these processes. We find it surprising however, that the system requires *four* redox active cofactors. This raises the possibility that E2 is involved in other reactions involved with the metabolism of lactate or acrylate. This point is currently under investigation.

ACKNOWLEDGMENTS

We thank Bob Vaillancourt for his assistance in metal analyses and Louise Robichaud for help in preparing the manuscript. The continued advice and support of Dr. B. L. Vallee is gratefully acknowledged.

Registry No. O_2, 7782-44-7; CoA, 85-61-0; acrylyl-CoA, 5776-58-9; lactyl-CoA, 1926-57-4; crotonyl-CoA, 992-67-6; propionyl-CoA, 317-66-8; lactyl-CoA dehydratase, 9031-12-3.

REFERENCES

Aasa, R., & Vanngard, T. (1975) *J. Magn. Reson. 19*, 308–315.

Beinert, H., & Thomson, A. J. (1983) *Arch. Biochem. Biophys. 222*, 333–361.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Chen, J., & Mortenson, L. E. (1977) *Anal. Biochem. 79*, 157–165.

Dowsing, R. D., & Gibson, J. R. (1969) *J. Chem. Phys. 50*, 294–303.

Emptage, M. H., Kent, T. A., Kennedy, M. C., Beinert, H., & Munck, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4674–4678.

Fendrich, G. (1983) Thesis, Brandeis University, Waltham, MA.

Hagan, W. R. (1981) *J. Magn. Reson. 44*, 447–469.

Kuchta, R. D., & Abeles, R. H. (1985) *J. Biol. Chem. 260*, 13181–13189.

Laemlli, V. K. (1970) *Nature (London) 227*, 680–685.

Latimer, W. M. (1964) in *Oxidation Potentials*, p 77, Prentice-Hall, Englewood Cliffs, NJ.

Lovenberg, W., Buchanan, B. B., & Rabinowitz, J. C. (1963) *J. Biol. Chem. 238*, 3899–3913.

Orme-Johnson, W. H., & Sands, R. H. (1973) in *Iron Sulfur Proteins* (Lovenberg, W., Ed.) Vol. 2, pp 195–238, Academic, New York.

Pierce, W. C., & Haenisch, E. L. (1940) in *Quantitative Analysis*, Wiley, New York.

Pilbrow, J. R. (1969) *Mol. Phys. 16*, 307–309.

Pilbrow, J. R. (1984) *J. Magn. Reson 58*, 186–203.

Pryce, J. D. (1969) *Analyst (London) 94*, 1151–1152.

Thiers, R. E. (1957) *Methods Biochem. Anal. 5*, 273–335.

Thomson, A. J. (1985) in *Metalloproteins, Part I* (Harrison, P. M., Ed.) pp 79–120, MacMillan, London.

Toy, A. D., Chaston, S. H. H., Pilbrow, J. R., & Smith, T. D. (1971) *Inorg. Chem. 10*, 2219–2225.

Vollmer, S. J., Switzer, R. L., & Debrunner, P. G. (1983) *J. Biol. Chem. 258*, 14284–14293.

Wahl, R. C., & Rajagopalan, K. V. (1982) *J. Biol. Chem. 257*, 1354–1359.

Wyard, S. J. (1965) *J. Sci. Instrum. 42*, 769–770.